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Virome of US bovine calf serum

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Abstract

Using viral metagenomics we analyzed four serum pools assembled from 715 calves in the United States. Two parvoviruses, bovine parvovirus 2 (BPV2) and a previously uncharacterized parvovirus designated as bosavirus (BosaV), were detected in 3 and 4 pools respectively and their complete coding sequences generated. Based on NS1 protein identity, bosavirus qualifies as a member of a new species in the copiparvovirus genus. Also detected were low number of reads matching ungulate tetraparvovirus 2, bovine hepacivirus, and several human papillomaviruses. This study further characterizes the diversity of viruses in calf serum with the potential to infect fetuses and through fetal bovine serum contaminate cell cultures.

Keywords: Metagenomics; parvovirus; bosavirus: phylogenetic.

Introduction

Fetal bovine serum is a potential source of viral contamination for cell cultures used in the production of biological products for human or animal use (Allander et al., 2001; Bauermann et al., 2013; Chen et al., 2008; Gagnieur et al., 2014; Marcus-Sekura et al., 2011; Nairn et al., 2003; Nims, 2006; Pinheiro de Oliveira et al., 2013) and is therefore routinely subjected to a range of virus-specific tests to ensure an absence of viral contaminations. While regulations list specific viruses whose absence must be confirmed, such as bovine viral diarrhea virus 1 and 2 and others, bovine viruses of concern beyond that list have been identified (Marcus-Sekura et al., 2011). Viral removal through filtration or inactivation methods may be used to reduce the risk of viral contamination; however, small non-enveloped viruses with ssDNA genomes, such as parvoviruses, are less susceptible to such measures. Using an unbiased metagenomics approach we characterized the viral sequences present in pools of bovine serum samples collected from calves in the US. The ability of these parvoviruses to contaminate fetal bovine serum remains to be determined.

Materials and Methods

Four bovine sera pools collected from different areas of the United States were analyzed (**Table 1)**. These samples were collected as part of a hepatitis E virus seroepidemiological study. The 25 calves in GA1 group and 90 calves in GA2 were sampled at 5-6 time points. The 375 calves in IA2 group and the 225 cows from VT1 group were sampled once. VT1 group from the Virginia Tech dairy herd, included animals from the lactating dairy herd (various lactation and days in milk) as well as pregnant heifers.

Library preparation and computational analysis were performed as previously described (Li et al., 2015; Zhang et al., 2016). Briefly, serum pools were filtered through a 0.45-μm filter (Millipore) to remove eukaryotic- and bacterial cell–sized particles, and 330 μL of each pool was then subjected to a mixture of nuclease enzymes to reduce the concentration of free (non-viral encapsidated) nucleic acids (Zhang et al., 2016). Viral nucleic acids were then extracted (MagMAX Viral RNA Isolation Kit, Ambion, Inc, Austin, Tx, USA) and random RT-PCR was used to amplify RNA and DNA. Four libraries were constructed using Nextera XT DNA Sample Preparation Kit (Illumina) and sequenced using the Miseq Illumina platform with 250 bases paired ends with dual barcoding for each pool.

Results

Four serum pools from a total of 715 animals were enriched for viral nucleic acids which were then randomly amplified and deep sequenced on the Ilumina platform. Out of ~47.4 million sequence reads, ~ 1% (44,279) were found by BLASTx to contain open reading frames encoding for parvovirus related proteins (BLASTx E scores $<10^{-5}$). Two complete coding regions of parvovirus genomes could be assembled. Bovine parvovirus 2 (BPV2 in the ungulate copiparvovirus 1 species) was detected and its genome assembled (GenBank accession number KY019140). BPV2's nonstructural (NS) and VP1 proteins showed 94-96 and 89-96 % identity to the 3 BPV2 genomes currently available in GenBank database. BPV2 sequences were detected in three pools (Table 1). The second parvovirus genome was more divergent relative to known genomes showing its closest relative to be porcine parvovirus 6 (PPV6 in the ungulate copiparvovirus 2 species) with 40% amino acid identity in their non-structural protein. The virus was named bosavirus (Bovine serum associated virus deposited as GenBank accession number KY019139). Phylogenetic analyses showed bosavirus' NS clustering with the NS of copiparvoviruses (Fig 1). The bosavirus VP was slightly more closely related to the VP of the recently described sesavirus (36% identity) from a sea lion than to that of PPV6 (34% identity) (Fig 2). Bosavirus sequences could be detected in all four pools (Table 1). According to a proposal from the International Committee on Taxonomy of Viruses (ICTV), members of the same parvovirus genus should share >30 %

identity in NS1 while members of the same species should exhibit >85% identity in NS1 (Cotmore et al., 2014). Based on pair-wise NS1 alignments, bosavirus is therefore proposed as member of a new species in the *Copiparvovirus* genus (ungulate copiparvovirus 3).

Using the bosavirus and the BPV2 genomes described above and the program Genious (Genious R6, 5.6.3 software with default settings) we calculated that 98.21% of these sequences could be matched to the bosavirus genome and 1.28% to BPV2 genome. Therefore 99.5% of parvovirus-related protein sequences detected here belonged to these two parvovirus species. The remaining parvovirus related reads consisted of mutated BPV2 and bosavirus reads (likely due to sequencing errors), chimeric reads that were only partly BPV2 or bosavirus, and five reads in GA2 pool that matched (>95% nucleotide similarity) porcine hokovirus (GenBank EU200677) (Lau et al., 2008) in the ungulate tetraparvovirus 2 species (Cotmore et al., 2014).

Other viral sequences were also detected but in much smaller numbers. Five sequence reads showing 97-100% amino acid sequence identity to the RNA genome of bovine hepacivirus a recently described member in the *Hepacivirus* genus of the *Flaviviridae* family (GenBank: KP265948.1) (Corman et al., 2015; Smith et al., 2016) were detected in GA2 pool. Bovine hepacivirus RNA has been detected in 1.6% of individually tested cows and in 3.8% of tested herds in Germany where its tissue distribution indicated possible liver tropism but no clear association with disease has been established (Baechlein et al., 2015). Also detected were 4 papillomavirus reads. The GA2 pool yielded one read that was 100% identical to human betapapillomavirus HPVX14 (GenBank: AF054874.1). Pool IA2 yielded three papillomavirus reads. One was 91% identical to the unclassified human papillomavirus type 174 (GenBank: HF930491.1), one 98% identical to human betapapillomavirus RTRX7 (GenBank: U85660.1) and one 96% identical to human betapapillomavirus type 151 (GenBank: FN677756.1).

Discussion

Our analysis characterizing enriched viral sequences in calf serum showed that parvovirus sequences dominated relative to other viruses (Table 1). As bovine fetuses acquire their viral infections from pregnant cows the potential exist for viruses described to also infect fetuses. The detection of a previously uncharacterized parvovirus (bosavirus) present in each US serum pool analyzed indicates that its presence and infectivity should be considered when testing fetal bovine serum for viral contamination. To date, members of the *Copiparvovirus* genus (classified based on NS) have only been reported in bovine or porcine samples (Cotmore et al., 2014). Whether only ungulates can be infected by copiparvoviruses, therefore posing little risk of infection to non-ungulate mammals, will become clearer as more parvoviruses are described in additional mammalian species. While bosavirus can be classified as a copiparvovirus based on its NS, its VP was more closely related to that of a parvovirus from the feces of a carnivore (Zalophus californianus or California sea lion) whose NS sequence falls outside the range of copiparvoviruses (Phan et al., 2015). The tropism of bosavirus may therefore extend beyond that of the currently known copiparvoviruses so far described only in ungulate samples. A few reads of porcine hokovirus , classified in the *Tetraparvovirus* genus, were also detected in one pool indicating that this virus, previously reported in porcine samples, may also infect calves albeit at low level relative to bosavirus and BPV2 (Table 1).

A low number of bovine hepacivirus (*Flaviviridae* family), a virus originally detected in African cattle and then herds in Germany, were also detected in one pool (Baechlein et al., 2015; Corman et al., 2015). Whether this hepacivirus can be transmitted to bovine fetuses or is capable of infecting other species is not known. Another member of the *Flaviviridae* family, bovine viral diarrhea virus in a different genus (*Pestivirus*) was not detected in our study. The absence of BVDV detection may be due to the high rate of BVDV vaccination in US herds and low rate of persistent viremia (Fulton et al., 2009; Wittum et al., 2001). The papillomavirus sequences detected were closely related to viruses detected in human skin. Whether these sequences reflect contamination with human skin or bovine papillomaviruses possibly introduced into the plasma pools from calve skin plugs during phlebotomy is also unknown (Buchta et al., 2005; Patton and Schmitt, 2010).

The vast majority of viral reads therefore originated from BPV2 and bosavirus whose infectivity to other species is unknown. Parvoviruses are particularly difficult to remove by filtration, likely due to small capsid sizes of 20-30nm (Caballero et al., 2014; Gefroh et al., 2014; Kwon et al., 2014). Based on viral particle size, the 100nm pore filtration step used in the manufacture of fetal bovine serum is not expected to completely remove parvoviruses. The small ssDNA genomes of ~5Kb may also make parvoviruses resistant to different viral nucleic acid inactivation methods (Caballero et al., 2014; Gefroh et al., 2014; Kwon et al., 2014). The FDA mandated testing for the detection of extraneous viruses (9 CFR 111.47) includes serological tests for parvovirus antigens of bovine parvovirus (*Bocaparvovirus* genus), canine parvovirus (*Protoparvovirus* genus), feline panleukopenia virus (*Protoparvovirus* genus), and porcine parvovirus (*Protoparvovirus* genus), following tissue culture infections. Based on the high degree of genetic divergence of these parvoviruses to BPV2 and bosavirus (both in *Copiparvovirus* genus) strong serological cross reactivity is unlikely.

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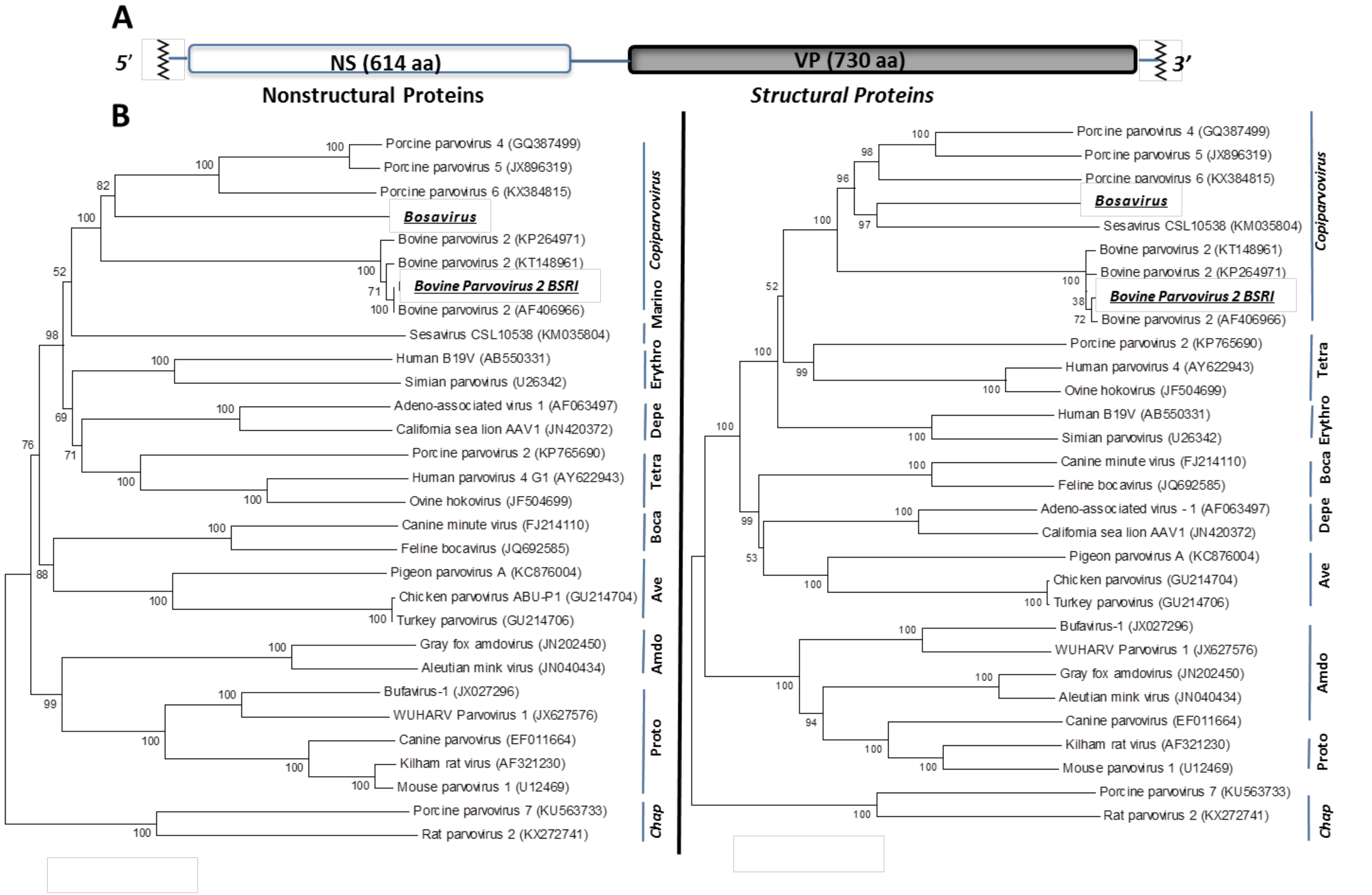
Figure

Figure 1.
Genome structure of novel parvovirus reported in this study. Maximum likelihood phylogenies are showing the relationship of the novel Bosavirus and BPV2 (A and B). Phylogenetic trees generated with NS and VP of Bosavirus and the representatives of ten genera in the subfamily *Parvovirinae*.

Figure 2.
VP and NS amino acid sequences identities between Bosavirus and Copiparvoviruses. The lower-left triangle shows homology between VP1 protein sequences of the six Copiparvoviruses generated by ClustalW alignment using Geneious Pro 6.1.8 software with default settings. The upper-right triangle shows NS amino acid sequence identities against of the six Copiparvoviruses.


A
5′
NS (614 aa)
VP (730 aa)
3′
Nonstructural Proteins
Structural Proteins
B
Porcine parvovirus 4 (GQ387499)
Porcine parvovirus 5 (JX896319)
Porcine parvovirus 6 (KX384815)
Bosavirus
Bovine parvovirus 2 (KP264971)
Bovine parvovirus 2 (KT148961)
Bovine Parvovirus 2 BSRI
Bovine parvovirus 2 (AF406966)
Sesavirus CSL10538 (KM035804)
Human B19V (AB550331)
Simian parvovirus (U26342)
Adeno-associated virus 1 (AF063497)
California sea lion AAV1 (JN420372)
Porcine parvovirus 2 (KP765690)
Human parvovirus 4 G1 (AY622943)
Ovine hokovirus (JF504699)
Canine minute virus (FJ214110)
Feline bocavirus (JQ692585)
Pigeon parvovirus A (KC876004)
Chicken parvovirus ABU-P1 (GU214704)
Turkey parvovirus (GU214706)
Gray fox amdovirus (JN202450)
Aleutian mink virus (JN040434)
Bufavirus-1 (JX027296)
WUHARV Parvovirus 1 (JX627576)
Canine parvovirus (EF011664)
Kilham rat virus (AF321230)
Mouse parvovirus 1 (U12469)
Porcine parvovirus 7 (KU563733)
Rat parvovirus 2 (KX272741)
Copiparvovirus
Marino
Erythro
Depe
Tetra
Boca
Ave
Amdo
Proto
Chap
Porcine parvovirus 4 (GQ387499)
Porcine parvovirus 5 (JX896319)
Porcine parvovirus 6 (KX384815)
Bosavirus
Sesavirus CSL10538 (KM035804)
Bovine parvovirus 2 (KT148961)
Bovine parvovirus 2 (KP264971)
Bovine Parvovirus 2 BSRI
Bovine parvovirus 2 (AF406966)
Porcine parvovirus 2 (KP765690)
Human parvovirus 4 (AY622943)
Ovine hokovirus (JF504699)
Human B19V (AB550331)
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Mouse parvovirus 1 (U12469)
Porcine parvovirus 7 (KU563733)
Rat parvovirus 2 (KX272741)
Copiparvovirus
Tetra
Erythro
Boca
Depe
Ave
Amdo
Proto
Chap

		NS						
		Bosavirus	Sesavirus	Porcine parvovirus 4	Porcine parvovirus 5	Porcine parvovirus 6	Bovine parvovirus 2 BSRI	Bovine parvovirus 2
VP	**Bosavirus**	100	22.9	32.4	32.6	40	30.3	30
	Sesavirus CSL10538 (KM035804)	36	100	24.9	25.5	25.2	25.3	25
	Porcine parvovirus 4 (GQ387499)	32	32.9	100	86.1	53.1	33.4	33
	Porcine parvovirus 5 (JX896319)	27	25.2	53.2	100	51.9	33	33
	Porcine parvovirus 6 (KX384815)	34	22.6	38.6	30.6	100	30.8	31
	Bovine parvovirus 2 BSRI 2016	24	23.1	29.8	25.8	20.2	100	93
	Bovine parvovirus 2 (AF406966)	24	23.2	29.8	25.8	20.2	96.1	100

Table 1. Characteristics of the bovine plasma pools analyzed in this study including viruses identified by NGS. Four sera pools approximately 1 mL collected from different areas of the United States were applied for NGS.

Pool name	Animal number	Age	Sampling days	Geographic area (US States)	Total number of reads	Virus detected (Reads)
GA1	25	3-240 days	3,30,60,120,200,240	GA	7.727.432	1. Bosavirus (30,590)
GA2	90	3-240 days	3,30,60,120,200,240	GA	14.190.694	1. Bosavirus (1,931) 2. Bovine parvovirus 2 (286) 3. Bovine hepacivirus (5) 4. Human papillomavirus (4) 5. Porcine hokovirus (5)
IA2	375	Unknown	Unknown	IA,NE, SD,NM, OK,TX, SD,ND,WY, MT.	11.227.852	1. Bosavirus (9,980) 2. Bovine parvovirus 2 (106) 3. Human papillomavirus (3)
VT1	225	Unknown	All ages and all stages of lactation	VA	12.291.250	1. Bosavirus (989) 2. Bovine parvovirus 2 (184)